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Anabaena circadian clock proteins KaiA and KaiB reveal a potential common binding site to their partner KaiC

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The cyanobacterial clock proteins KaiA and KaiB are proposed as regulators of the circadian rhythm in cyanobacteria. Mutations in both proteins have been reported to alter or abolish circadian rhythmicity. Here, we present molecular models of both KaiA and KaiB from the cyanobacteria *Anabaena* sp PCC7120 deduced by crystal structure analysis, and we discuss how clock-changing or abolishing mutations may cause their resulting circadian phenotype. The overall fold of the KaiA monomer is that of a four-helix bundle. KaiB, on the other hand, adopts an alpha–beta meander motif. Both proteins purify and crystallize as dimers. While the folds of the two proteins are clearly different, their size and some surface features of the physiologically relevant dimers are very similar. Notably, the functionally relevant residues Arg 69 of KaiA and Arg 23 of KaiB align well in space. The apparent structural similarities suggest that KaiA and KaiB may compete for a potential common binding site on KaiC.

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Introduction

Circadian rhythms, biological oscillations with a period of approximately 24 h, have been observed in a wide variety of organisms from cyanobacteria to green plants to humans (Dunlap *et al*, 1999; Mori and Johnson, 2001; Young and Kay, 2001). In order for biological oscillations to be denoted as a circadian 'clock', they have to fulfil three criteria. They must: (1) persist in the absence of external time cues; (2) reset their phase by exposure to light or dark; and (3) compensate for temperature changes during the circadian period. It has been postulated that the circadian clock controls the rhythm that is fundamental for cells to adapt to the alternation between day and night (Pittendrigh, 1993). Physiological studies have identified several clock-related genes in cyanobacteria, *Neurospora*, *Arabidopsis*, *Drosophila*, and mammals (Dunlap, 1999; Young and Kay, 2001).

Cyanobacteria are the simplest organisms known to possess a circadian clock. They are well suited as genetic and physiological model systems for the study of circadian rhythms (Iwasaki and Kondo, 2000; Mori and Johnson, 2001). Various cyanobacterial strains, containing a bioluminescence reporter gene, have been used to monitor circadian rhythms and identify key genes involved in its regulation and maintenance (Kondo *et al*, 1993; Ishiura *et al*, 1998). Using genetic complementation of various rhythm mutants, a gene cluster *kai* composed of three genes (*kaiA*, *kaiB*, and *kaiC*) was identified and cloned from the cyanobacterium *Synechococcus* sp PCC7942 (Ishiura *et al*, 1998). *Kai* genes, their name derived from the Japanese word for cycle, are found ubiquitously among cyanobacteria (Lorne *et al*, 2000). Amino-acid sequence analyses have shown that the Kai proteins display no homology to clock proteins identified in other organisms (Dunlap *et al*, 1999). However, circadian rhythms can be altered or even abolished through mutations in any of the *kai* genes and, as a corollary, re-introduction of the *kai* gene cluster will restore lost rhythmicity (Ishiura *et al*, 1998). Evidence gathered so far indicates that all the three Kai proteins play a crucial role for circadian rhythm in cyanobacteria.

How exactly the *kai* genes generate and maintain rhythm in cyanobacteria is only slowly being unravelled. Transcriptional analysis of the *kai* genes has provided early clues on how this system functions (Ishiura *et al*, 1998). Two promoters control *kai* gene transcription: P_{kaiA} for *kaiA* and P_{kaiBC} for *kaiB* and *kaiC*. Both promoters regulate the rhythm of the same phase for their respective mRNAs. Overexpression of the *kaiC* gene will immediately and completely repress its own P_{kaiBC} promoter. This strong negative autoregulation suggests that KaiC functions as the key component of the circadian oscillation in cyanobacteria. However, inactivation of P_{kaiA} reduces P_{kaiBC} activity, while overexpression of KaiA dramatically increases P_{kaiBC} activity. As P_{kaiA} is in phase with P_{kaiBC}, it appears that KaiA regulates the expression of *kaiBC* in a positive manner.

Recently, the structure of the N-terminal domain of KaiA, termed KaiA 135N, from *Synechococcus elongatus* sp PCC 7942 has been determined using NMR techniques (Williams *et al*, 2002). KaiA 135N adopts the structure of a pseudo-receiver domain and is postulated to act as a regulatory component of KaiA activity. It should be noted that the sequence of *kaiA* is the one least conserved among the *kai* genes. KaiA protein from *Anabaena* sp PCC 7120, one of the objects of this study, is highly homologous to the C-terminal

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part of the *S. elongatus* KaiA, but completely lacks the region corresponding to KaiA 135N. The conserved portion of KaiA has been reported to stimulate KaiC phosphorylation (Iwasaki *et al*, 2002; Williams *et al*, 2002; Kitayama *et al*, 2003). Also KaiA mutations in *S. elongatus* appear to lower P_{kaiBC} expression (Ishiura *et al*, 1998; Nishimura *et al*, 2002), suggesting a dual role for KaiA in regulating the length of the period of the circadian oscillation.

In contrast, KaiB, which shares its promoter with KaiC, has been identified as an attenuator of KaiC phosphorylation (Kitayama *et al*, 2003). Again setting it apart from KaiA, the primary sequence of KaiB is well conserved among cyanobacterial species. Both KaiB and KaiC protein levels cycle rhythmically even under constant light conditions (Xu *et al*, 2000). As KaiB and KaiC are co-transcribed, the phasing of their protein levels suggests that both proteins have similar turnover rates. While KaiB–KaiC interactions are well documented, the evidence for KaiA–KaiB interactions is much weaker (Iwasaki *et al*, 1999). KaiB point mutations show a shortened circadian period likely arising from its diminished ability to reduce KaiC phosphorylation (Ishiura *et al*, 1998).

Based on a genomic survey, KaiC from *S. elongatus* sp PCC7942 was proposed to be a member of the bacterial RecA/DnaB family (Leipe *et al*, 2000). Also, analysis of the amino-acid sequence of KaiC shows a significant similarity between the first and second halves of the sequence, with each half containing a trinucleotide-binding Walker A motif (Ishiura *et al*, 1998; Walker *et al*, 2000). Mutations of these Walker A motifs abolish nucleotide binding and rhythmicity is severely disrupted or abolished (Nishiwaki *et al*, 2000; Mori and Johnson, 2001). Characterized by biophysical methods and electron microscopy (EM), KaiC is reported to form hexameric rings in the presence of ATP (Mori *et al*, 2002). In line with its apparent negative feedback on P_{kaiBC}, KaiC was able to bind onto forked DNA substrates despite lacking any known DNA-binding motifs (Mori *et al*, 2002). In addition, KaiC undergoes autophosphorylation *in vitro* (Nishiwaki *et al*, 2000), a feature that may play a role in its functional regulation and/or degradation, as is seen for eukaryotic clock proteins FRQ and PER (Young and Kay, 2001). Point mutations in KaiC can cause a wide variety of period changes including shortened and extended periods as well as complete loss of rhythmic cycling (Ishiura *et al*, 1998). While KaiC appears to be the time-keeping protein for the circadian rhythm, its partners, KaiA and KaiB, seem to be crucial for adjusting the periodicity of the level of KaiC protein, thereby compensating for length changes in day/night cycles.

In vitro, *S. elongatus* Kai proteins form oligomeric complexes in all possible combinations (Iwasaki *et al*, 1999). *In vivo*, homodimeric interactions between KaiB molecules have been demonstrated using a bioluminescence resonance energy transfer system (Xu *et al*, 1999). Pull-down assays demonstrated that each Kai protein was able to interact with each of the other two Kai proteins, although the interaction between KaiA and KaiB was quite weak (Iwasaki *et al*, 1999). Moreover, KaiC appears to have dual sites (one in each of the internal repeats) that have the ability to bind onto KaiA and KaiB proteins (Iwasaki *et al*, 1999). Further *in vivo* studies support the formation of higher order complexes composed of all three Kai proteins, which vary in size depending on subjective day and night times (Kageyama *et al*, 2003).

Although our knowledge of the regulation and binding interactions of the Kai proteins has been growing recently, very little is known about the molecular structures of the individual proteins. In an attempt to further the structural characterization of the Kai system, we now report the three-dimensional structures of both KaiA and KaiB from the cyanobacterium *Anabaena* sp PCC7120 and discuss their potential interactions and functions.

Results

Determination of the oligomerization state of KaiA and KaiB

Following purification, both recombinant KaiA and KaiB proteins were loaded in separate experiments onto a Superdex 200 column for size fractionation. KaiA eluted within a single peak of about ~27 kDa, which is close to the estimated size of the KaiA dimer. On reducing SDS–PAGE gels, KaiA ran as a monomer showing a band around 14 kDa in size, and no contaminating bands could be detected by Coomassie Blue staining. KaiB eluted from the column in fractions corresponding to a molecular weight of about ~30 kDa, which again is close to the estimated size of the KaiB dimer. On a reducing SDS–PAGE gel, peak fractions ran as monomers around 15 kDa in size, with no other bands visible. These findings are consistent with reports that KaiA and KaiB form homodimers *in vivo* (Kageyama *et al*, 2003).

Crystallization of native KaiA, native KaiB, and KaiB mutants

Native KaiA crystals were reproducibly grown in 100 mM MES (pH 6.0) and using 1.1 M $MgSO_4$ as a precipitant. They were strongly birefringent and displayed apparent six-fold symmetry along their major axis. The crystals belonged to space group $P6_522$ with cell axes $a = b = 57.3$ Å, $c = 137.5$ Å and one KaiA monomer per asymmetric unit. The crystal axes of the bromine soaked crystal changed slightly to $a = b = 57.1$ Å, $c = 137.5$ Å.

Native KaiB crystals could be reproducibly grown in 100 mM Tris (pH 7.5) using 18% (v/v) PEG 400 as a precipitant (Fluka). They belong to space group C2 with cell parameters of $a = 104.8$ Å, $b = 36.4$ Å, $c = 54.1$ Å, and $\beta = 98.8^\circ$. As wild-type KaiB contains only the N-terminal methionine, a residue often found to be mobile in crystals, mutants that contained an additional methionine residue to increase the probability of success of the MAD phase determination method were generated. Both KaiB mutants (L28 M and L69 M) required the use of higher concentrations of PEG400 (23.5% v/v) (Fluka) as well as the addition of 5% 1,6-hexanediol in order to form crystals. KaiB (L69 M) mutants typically grew as long rod-like crystals that had a strong tendency to split or fracture and therefore were not suitable for data collection. KaiB (L28 M) Se-Met crystals belonged to space group C2 with cell parameters of $a = 105.0$ Å, $b = 36.7$ Å, $c = 54.2$ Å, and $\beta = 99.1^\circ$.

Structure of KaiA

The crystal structure of KaiA was determined by the bromine-based MAD technique (Dauter and Dauter, 1999). As mentioned earlier, the KaiA protein of *S. elongatus* is nearly twice as large as the one from *Anabaena* sp PCC7120 caused by the

addition of an N-terminal domain of 180 amino acids (Figure 2), whose structure has recently been reported (Williams *et al*, 2002). Our analysis deals with the full-length KaiA protein from the closely related species PCC7120, which is highly homologous to the C-terminal domain of the former. The KaiA monomer adopts a four-helix bundle fold with the classical up–down–up–down arrangement of helices and a well-defined hydrophobic core buried between these helices (Carlacci and Chou, 1990) (Figure 1). Within the core, one finds several leucine and isoleucine side chains as well as tyrosine and phenylalanine rings. Residues at the N-terminus (1–7), although partially stabilized for some monomers by crystal contacts, appear to be mostly flexible as the electron density in that region is much less defined. In solution, this region of KaiA is likely free to move, but could adopt specific conformations upon interactions with KaiB, KaiC, or other protein-binding partners.

The asymmetric unit in the KaiA crystal lattice contains one monomer with a total of three dimers making up the unit cell. The dimer interface lies along one of the crystal axes of symmetry, with the majority of the interactions occurring along helices 3 and 4 (Figure 2). An inter-subunit disulphide bridge between subunits is formed between symmetry-related Cys 93 residues. In general, the dimer interface consists of predominantly hydrophobic interactions with a few backbone hydrogen bonds surrounding the hydrophobic region. They include the main chain atoms of Val 50*a* and Glu 90*b*, Ala 95*a* and Leu 88*b*, as well as their symmetry counterparts.

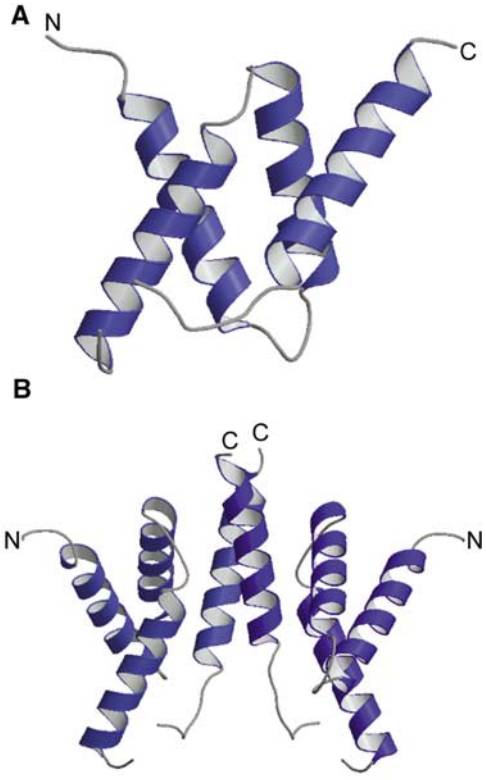

Figure 1 Structure of KaiA. (**A**) Ribbon diagram of monomer. Helices are coloured in blue. (**B**) Ribbon diagram of dimer. The two-fold axis runs vertically. Monomer A is coloured in light blue (for helices) while monomer B is coloured in darker blue. N- and C-termini are labelled.

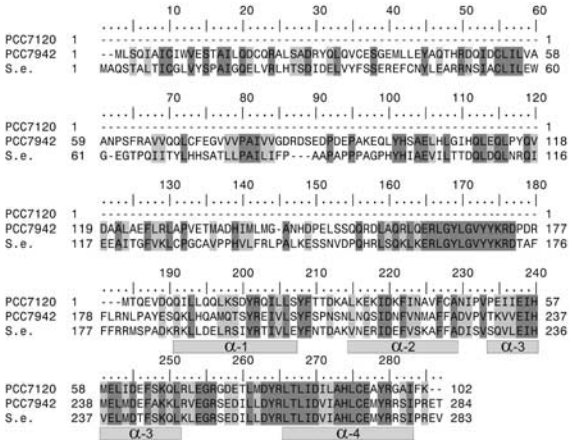

Figure 2 Alignments of the sequences of KaiA from *Anabaene* sp PCC7120, *Synechococcus* sp PCC7942, and *S. elongatus*. Identical residues across sequences are shaded by dark grey, while conserved residues are shaded by light grey (criteria defined by Clustal X). Hyphens indicate gaps. Secondary structural elements determined in the crystal structure are aligned with the corresponding sequence.

Mutations of KaiA in PCC7942 result in an extended circadian period or completely abolish rhythmicity in cyanobacteria (Ishiura *et al*, 1998; Nishimura *et al*, 2002). For PCC7942, the R249 H mutant of KaiA has been described as displaying an extended circadian period of 30 h (Ishiura *et al*, 1998). In a multiple sequence alignment, this residue is lined up with Arg 69 of the PCC7120 KaiA protein (Figure 2). The crystal structure places Arg 69 in a loop region between helices 3 and 4. One would expect that this mutation should not cause any major change in the internal structure of KaiA. It might, however, affect the way in which the two domains of PCC7942 KaiA interact or it could well be involved in binding to other partner proteins.

Another destructive mutation reported in KaiA of PCC7942 is E274 K (Ishiura *et al*, 1998). In PCC7120, the corresponding residue is Glu 94 (Figure 2). As this residue links the two subunits via a hydrogen bond, the resulting change in charge would obviously drastically weaken KaiA's ability to form dimers, the oligomerization state observed *in vivo* (Kageyama *et al*, 2003). It is easy to imagine how this defect could affect the stability or at least interfere with protein–protein interactions, leading to the observed abolishment of rhythmicity.

Structure of KaiB

The molecular model of KaiB was derived, applying the Se-Met-based MAD technique to the L28 M mutant. The overall fold of the KaiB monomer is a two α-helix/three β-sheet alpha–beta meander (Figure 3), with helical and sheet secondary structure elements alternating along the peptide chain (Figure 4). There is a loop region in each monomer that accounts for the majority of interactions between the monomers. It is flanked by Pro 51 and Pro 63. The first few residues of β-strand 3, Ile 88, and Leu 90 are also involved in the dimer interface, which consists of predominantly hydrophobic interactions coupled with some hydrogen bonding along the edges of the dimer interface between the main chain residues of Leu 53*a* and Ile 88*b*. An especially important residue in stabilizing the KaiB dimer seems to be Gln 52. Its side chain is

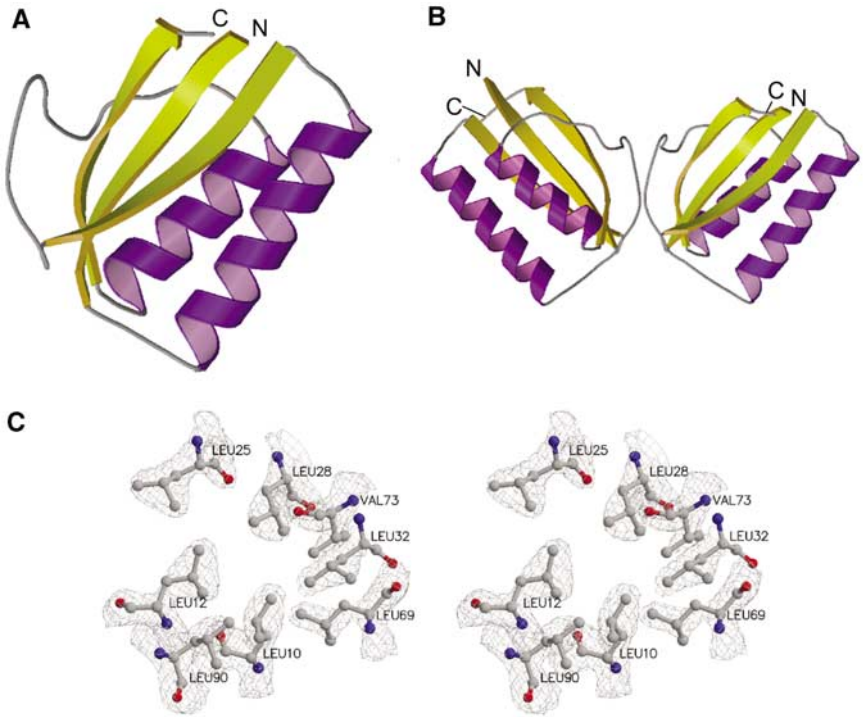

Figure 3 Structure of KaiB. (**A**) Ribbon diagram of monomer. Helices are coloured in purple while β-sheets are coloured in yellow. (**B**) Ribbon diagram of dimer. The two-fold axis runs vertically. Monomer A is coloured in purple and yellow (for helices and sheets), while monomer B is coloured in darker purple and gold. N- and C-termini are labelled. (**C**) Stereo view of the hydrophobic core of KaiB. Residues are labelled to the right and the electron density map is shown in light grey.

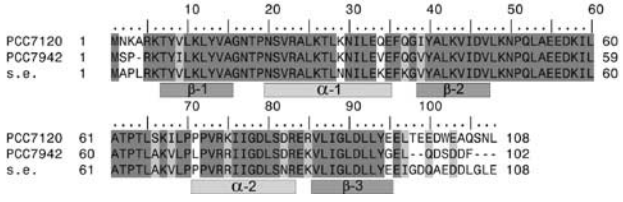

Figure 4 Alignments of the sequences of KaiB from *Anabaene* sp PCC7120, *Synechococcus* sp PCC7942, and *S. elongatus*. Identical residues across sequences are shaded by dark grey, while conserved residues are shaded by light grey (criteria defined by Clustal X). Hyphens indicate gaps.

involved in three hydrogen bond interactions linking it to the backbone carbonyls of Ser 81 and Leu 87, as well as the guanadinium group of Arg 85.

Leu 28, which was replaced by methionine in the KaiB (L28 M) mutant used for MAD phasing, is buried within the hydrophobic region between both α-helices and the β-sheet region. In contrast, Leu 69 is located adjacent to the second α-helix, its head group buried within a small hydrophobic pocket, surrounded by Leu 10, Ile 77, and Leu 90. Mutation of this residue into a methionine resulted in the growth of crystals of very poor quality. The mutation may cause α-helix 2 to shift due to the additional bulk of the methionine side chain. This, in turn, would affect the ability for dimers to pack tightly along the corresponding helices, thus introducing multiple defects into the growing crystal lattice.

Circadian period mutants of KaiB in PCC7942 have also been reported (Kondo *et al*, 1994). In one of them, Leu 11 is replaced with a phenylalanine, resulting in a phenotype with a shortened 21 h circadian cycle (Ishiura *et al*, 1998). This mutation maps onto Leu 12 of PCC7120 KaiB (Figure 4). The side chain of this amino acid resides directly in the centre of the large hydrophobic core of the KaiB monomer and is surrounded by residues from each of the secondary structure elements (Figure 4). The packing of the leucine and isoleucine side chains within this region is very well defined, suggesting that the packing is tight and well ordered. The space requirements of a flat aromatic ring are quite different from those of a branched side chain. Therefore, in a crowded environment, replacing leucine with a phenylalanine may lead to an overall rearrangement of the side chains, followed by a shift in the overall alignment of secondary structure elements. This, in turn, would certainly affect KaiB's ability to interact with its binding partners.

The second point mutation described for KaiB is R74W. It also results in a slightly shortened circadian rhythm of 22 h (Ishiura *et al*, 1998). Arg 74 of KaiB from PCC7942 maps onto Lys 75 of the PCC7120 protein (Figure 4). As the side chain of Lys 75 protrudes from the surface of the KaiB dimer, this point mutation should not cause any significant structural changes. Probably, this residue lies within a region involved in binding interactions to other proteins.

Co-precipitation of Kai protein complexes

Tests for physical association between KaiA or KaiB wild-type and mutant proteins with KaiC were performed *in vitro* using a binding assay analogous to those described in the literature (Iwasaki *et al*, 1999; Kageyama *et al*, 2003). We used Ni-NTA agarose (Invitrogen) beads as the specific protein precipitant to immobilize recombinant 6xHis-tagged KaiC, the only one of the Kai proteins modified in this way, and any associated protein complexes.

In the absence of 6xHis-tagged KaiC, neither wild-type KaiA nor KaiB proteins would bind to the Ni beads (Figure 7A, lanes 1, 6, 11, and 16). The tagged KaiC, however, bound readily (Figure 7A, lanes 2, 7, 12, and 17) and was eluted using high concentrations of imidazole. Wild-type KaiB protein could be pulled down by the Ni beads in the presence of KaiC, showing binding between KaiB and KaiC (Figure 7A). Increasing the molar ratio of KaiB:KaiC resulted in a saturation of KaiB bound to KaiC, with increasing amounts of unbound KaiB appearing in the flow through fractions (Figure 7A, lanes 13–15 and 18–20).

Unexpectedly, the presence of KaiA appeared to prevent KaiC from interacting with the Ni beads; consequently, KaiC appears in increasing amounts in the flow-through fractions as the KaiA:KaiC molar ratio is increased (Figure 7A, lanes 3–5 and 8–10). The ability of KaiA to cause KaiC's release from the beads explains why we were unable to see KaiA bound onto the resin even in the presence of KaiC. Loss of KaiC from the resin does indirectly confirm interaction between KaiA and KaiC proteins. Using a fixed concentration of KaiB while adding increasing quantities of KaiA showed a progressive loss of bound KaiC (Figure 7B, lanes 2–5 and 7–10), suggesting that KaiA interacts with KaiC, even in the presence of KaiB. Using increasing amounts of KaiB mixed with a fixed concentration of KaiA progressively rescued tagged KaiC's binding onto the beads taking along KaiB (Figure 7B, lanes 12–15 and 17–20). To provide evidence in a more direct manner, sequential competition-binding assays were also performed that showed that KaiA could be pulled down by pre-immobilized KaiC and could be competed off by subsequently adding KaiB and *vice versa* (see Supplementary data).

The R69A KaiA and R23A KaiB mutants were also tested for their ability to bind to KaiC and their behaviour was compared to that of the wild-type proteins (Figure 7C). Replacing Arg 69 of KaiA with an alanine produced a mutant protein that was no longer able to cause the release of KaiC from the Ni beads (Figure 7C, lanes 1–12), indicative of a loss of binding. Mutation of Arg 23 of KaiB to an alanine also had a significant effect, substantially reducing its affinity for KaiC (Figure 7C, lanes 13–24).

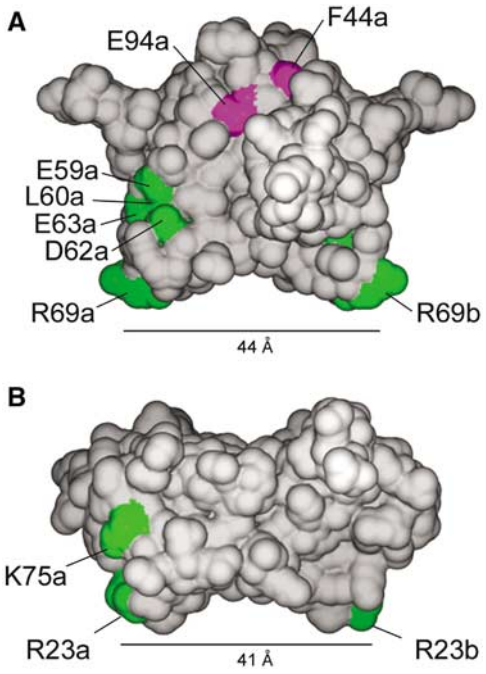

Figure 5 (**A**) Rendering of the solvent accessible surface of KaiA. Residues that, when mutated, extend or abolish the circadian rhythm are highlighted in green. Residues that, when mutated, affect *KaiBC* expression are highlighted in purple. Residues from one monomer are given the suffix a, while residues from the other monomer are given the suffix b. (**B**) Rendering of the solvent accessible surface of KaiB. Residues that, when mutated, decrease or abolish circadian rhythm are highlighted in green. Residues from one monomer are given the suffix a, while residues from the other monomer are given the suffix b. Both molecules are oriented with the two-fold rotation axis relating the two subunits located in the plane of the paper.

Discussion

Structural basis of KaiA and KaiB functions

In cyanobacteria, KaiC is a prime candidate for time-keeper of the circadian rhythm (Ishiura *et al*, 1998). KaiA is believed to be important in regulating KaiC turnover with the common and well-conserved domain of KaiA involved in stimulation of KaiC autophosphorylation (Iwasaki *et al*, 2002; Williams *et al*, 2002).

Size and amino-acid sequence of KaiB are also well conserved between cyanobacterial species. However, despite the identification of rhythmicity mutants of KaiB, relatively little is known about the actual mechanistic role played by KaiB in the maintenance of circadian rhythm. Recent data suggest that KaiB is an attenuator of KaiC phosphorylation (Kitayama *et al*, 2003). The crystal structures of KaiA and KaiB now shed some light on how KaiB mutants might affect its function, thus altering circadian rhythms in cyanobacteria.

Looking at the surfaces of the KaiA and KaiB dimers spanning their two-fold axis of symmetry, one discovers a striking similarity of shape and placement of some charged residues (Figure 5). If the two symmetry-related Arg 69 residues of the KaiA dimer are superimposed with the Arg 23 and Arg 23*b* residues of the KaiB dimer, the bulk of the molecules matches quite nicely (Figure 5). Especially, the curvature and rectangular footprint of that part of the molecular surface of KaiA and KaiB point to a possible common binding partner. We speculate that the arched surface, stretching between the two Arg 69 residues in the KaiA dimer and between the two Arg 23 residues in the KaiB dimer, may be where these dimers interact with KaiC. KaiA and KaiB appear to exert opposite effects on KaiC: KaiA promotes KaiC phosphorylation while KaiB attentuates it (Iwasaki *et al*, 2002; Kitayama *et al*, 2003). One compelling way to fulfil their respective functions would be for KaiA and KaiB to compete for the same binding site on KaiC. The importance of Arg 69 in KaiA is reflected by the loss of rhythmicity PCC7942 R249 H mutant (Nishimura *et al*, 2002), which would easily be explained by a change in the affinity of KaiA for its docking site on KaiC. The results of our binding assay (Figure 7C) support this interpretation as they show a drastic change in binding affinity between the equivalently located R69A mutant and wild-type forms of PCC7120 KaiA and KaiC.

Circadian period extending mutations in KaiA have been shown to cluster along residues 239–245 in PCC7942 and typically involve the replacement of charged side chains by uncharged ones (Nishimura *et al*, 2002). The corresponding

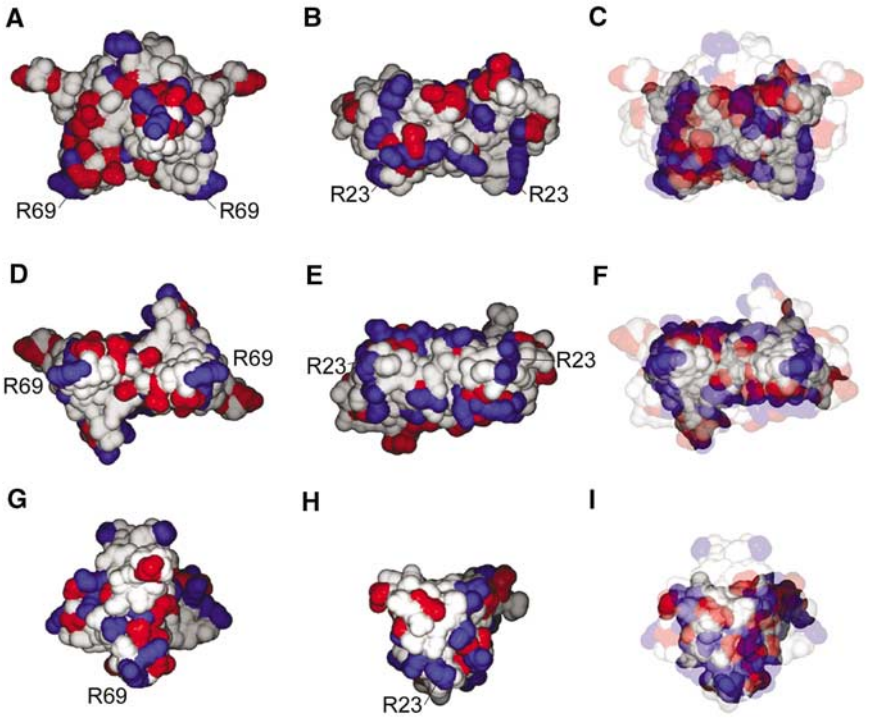

Figure 6 Surface potential comparisons between KaiA and KaiB dimers using SPOCK with each of the proteins at 50% transparency: brighter regions represent physical overlap. (**A**) KaiA dimer in the same orientation as in Figure 1B. Arg 69 of the monomers is located in the blue region at the bottom of the surface plot. (**B**) KaiB dimer in the same orientation as in Figure 3B. Arg 23 of the KaiB monomers is located in the blue region at the 'bottom' of the surface plot. (**C**) Overlay of (A) and (B). (**D**) KaiA dimer 'bottom' surface. (**E**) KaiB dimer 'bottom' surface. (**F**) Overlay of (D) and (E). (**G**) KaiA dimer side surface. (**H**) KaiB dimer side surface. (**I**) Overlay of (G) and (H).

residues in the PCC7120 KaiA model are Glu 59, Leu 60, Glu 63, and Asp 62. They flank the sides of the KaiA dimer close to Arg 69 (Figure 6). While the geometry of the residues flanking Arg 23 in KaiB is similar to that found in KaiA, their chemical nature is quite different. This mixture of conserved and deviating features would well explain the common affinity displayed by KaiA and KaiB for KaiC. Whether the phosphorylation state of KaiC is directly related to circadian rhythms remains to be determined.

Mutations of KaiA (F224S and E274 K of PCC7942) that alter *KaiBC* expression were believed to be located at other regions of KaiA (Nishimura *et al*, 2002). This assignment has now been confirmed. The mutation sites correspond to Phe 44 and Glu 94 in KaiA from PCC7120, both of which are located at the surface of KaiA opposite from the Arg 69 residues. This finding leads us to believe that the two reported functions of KaiA, interaction with DNA and regulation of autophosphorylation of KaiC, are performed by opposite regions on the KaiA dimer. As overexpression of KaiA has been shown to increase P_{kaiBC} activity (Ishiura *et al*, 1998), perhaps this region of KaiA may have affinity for the promoter region or some currently unidentified transcription factor specific to the P_{kaiBC} region. While recent studies have shown that P_{kaiBC} is not absolutely necessary for maintaining circadian rhythmicity in cyanobacteria, they do not rule out the importance of P_{kaiBC} upregulation by KaiA under normal conditions in response to external stimuli (Xu *et al*, 2003).

Given the high degree of sequence identity between corresponding proteins from PCC7942 and PCC7120, we feel fully justified in discussing the findings of mutational analyses obtained with PCC7942 proteins in the context of our structural results. To test whether KaiA and KaiB may be competing for a common binding site on KaiC, we performed *in vitro* binding assays. Our binding assay using purified KaiA, KaiB, and 6xHis-tagged-KaiC does agree with a competitive nature of wild-type KaiA and KaiB on KaiC *in vitro* (Figure 7A). Unexpectedly, incubation with increasing amounts of KaiA led to a corresponding reduction of binding of 6xHis-tagged-KaiC to the Ni beads (Figure 7A, lanes 2–5 and 7–10). Potential explanations for this finding are the masking of the His-tag either directly through binding of KaiA or via induction of a conformational change. As pre-incubation of KaiC with Ni beads followed by incubation with KaiA allowed co-elution of KaiA and KaiC, the former seems to be a more probable interpretation (see Supplementary data). KaiA binding to KaiC in this assay could subsequently be competed off using KaiB. Point mutations located at the postulated KaiC interaction sites on both KaiA and KaiB proteins were also generated to test their effects on binding; Arg 69 of KaiA and equivalently placed Arg 23 of KaiB were changed to alanine residues. As expected, the mutations did not affect these proteins' abilities to dimerize (data not shown), but strongly affected their binding affinities to KaiC. Whereas binding of KaiA (R69A) seems to be completely abolished (Figure 7C, lanes 1–3, 7–9), KaiB (R23A) still binds to KaiC albeit with reduced affinity (Figure 7C, lanes 13–15 and 19–21). These results are consistent with our assignment of the concave surface, common to KaiA and KaiB, representing the respective binding sites with KaiC. Despite the competitive nature of KaiA and KaiB binding to KaiC, as seen in our assay, these binding experiments alone cannot prove that KaiA and KaiB specifically bind onto the same site on KaiC. A conforma-

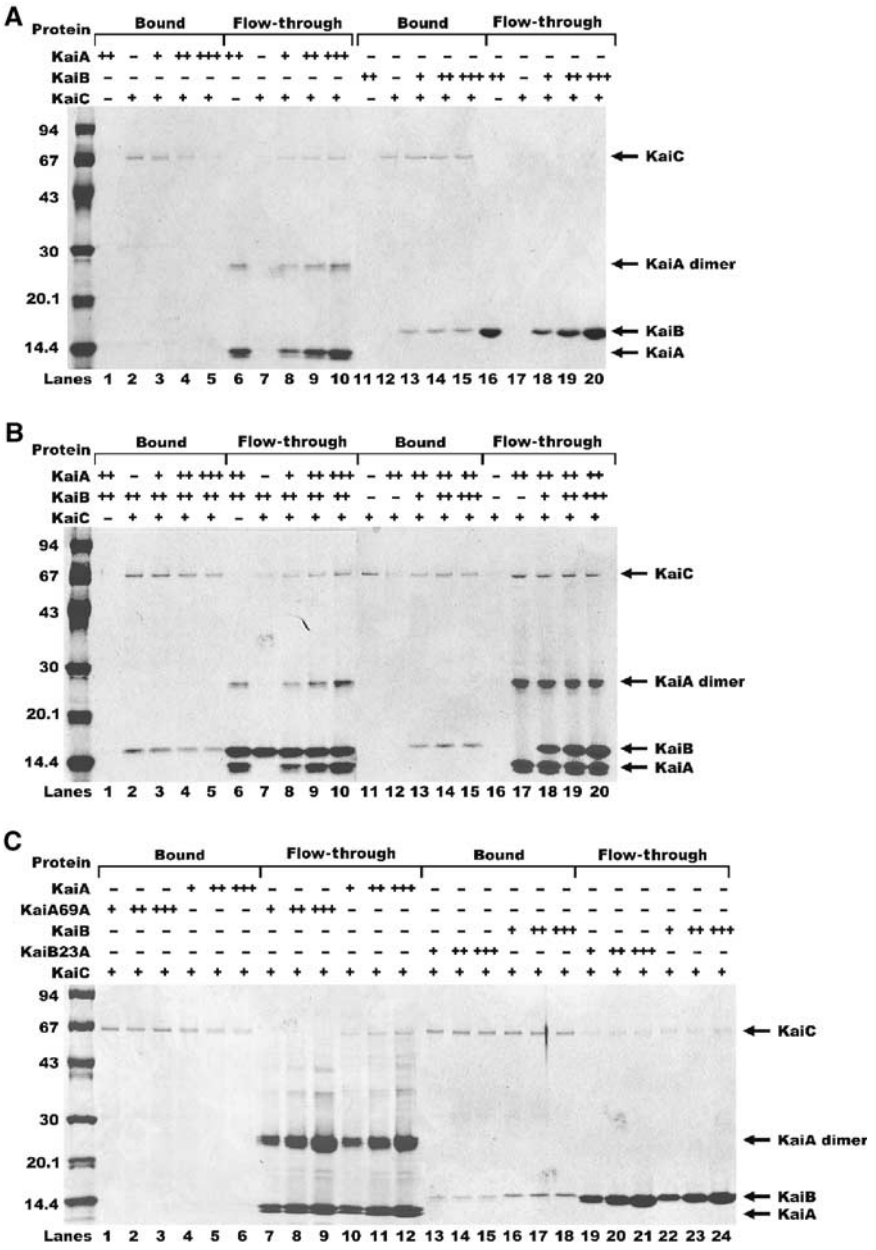

Figure 7 Interactions between KaiC and KaiA, KaiB, KaiA69A, and KaiB23A proteins. Ni beads were incubated with the various protein mixtures. Proteins that did not interact with the Ni beads were collected and loaded in the lanes labelled as Flow-through. Proteins that bound to the Ni beads were then eluted using 1 M imidazole and loaded in the lanes labelled 'Bound'. Known protein bands are indicated by arrows on the right side of the gels; an intersubunit disulphide bond of KaiA is quite resistant to reduction by β-mercaptoethanol, resulting in the persistence of dimers. (**A**) Binding profiles of wild-type KaiA or KaiB to a fixed amount of 6x-His-tagged KaiC bound to Ni beads. The presence of a particular protein in a given solution is indicated by '+' signs, and its absence by '−' signs. '+': 40 μg of protein was added; '++': 80 μg of protein was added; and '+++': 160 μg of a particular protein was added. Equal volumes of sample were loaded in each lane. Lanes are numbered below. (**B**) Competition binding profiles of increasing amounts of KaiA competing with a fixed amount of KaiB, or *vice versa*, on a fixed amount of 6x-His-tagged KaiC bound to Ni-beads. (**C**) Comparison of binding profiles of mutant and wild-type KaiA and KaiB proteins to 6x-His-tagged KaiC bound to Ni beads.

tional change of KaiC due to KaiA and KaiB binding as well as partial overlap of their respective binding sites could also be used to explain the current observations without, however, being able to explain the remarkable fit of the size and shape of the corresponding concave molecular surfaces.

The status of KaiC phosphorylation appears to be key to KaiC's ability to bind to the *kaiBC* promoter (Iwasaki *et al*, 2002). KaiC shows internal sequence duplication (Iwasaki *et al*, 1999) and forms hexameric rings in an ATP-dependent manner (Mori *et al*, 2002). One can imagine KaiA and KaiB dimers competing for binding sites along the outer edges of the KaiC hexamer, affecting KaiC's autophosphorylation activity, thus regulating KaiC function. Recently, Vakonakis *et al* (2004) proposed a model of a KaiA–KaiC complex consistent with such an interpretation. Autophosphorylation mixing experiments on KaiC in the presence of KaiA and KaiB showed that KaiC's autophosphorylation activity is markedly increased by the presence of KaiA, but that the level of KaiC

activity dropped by half if KaiB was present. KaiB alone did not effect KaiC autokinase activity (Kitayama *et al*, 2003). This evidence, together with molecular structures of KaiA and KaiB, agrees well with a model of competition between KaiA and KaiB for a common binding site on KaiC.

While having no known enzymatic activity on their own, KaiA and KaiB are an intrinsic part of the cyanobacteria circadian system, their function apparently arising from their ability to affect KaiC phosphorylation rates (Williams *et al*, 2002; Kitayama *et al*, 2003). In conclusion, we have determined the crystal structures of both KaiA and KaiB from *Anabaena* sp PCC7120. The atomic models suggest how specific point mutations of KaiA and KaiB could lead to changes in the structures of these molecules, thereby interfering with their roles in establishing proper circadian rhythm. They are also in favour of a structure-based competition model between KaiA and KaiB for a shared binding site on KaiC. However, understanding the exact mechanism through which KaiA and KaiB affect KaiC phosphorylation will require a high-resolution structure of KaiC or, even better, of its complexes with KaiA and KaiB.

Materials and methods

Cloning and mutagenesis

Wild-type KaiA, KaiB, and KaiC genes were cloned by polymerase chain reaction (PCR) from *Anabaena* sp PCC7120 genomic DNA. The full-length KaiA gene was amplified using a 5′ primer (5′ CGCGCCATGGCTCAAGAAGTTGATCAGCAAATATTATTG) and a 3′ primer (5′ CGCGGGATCCTTATTTAAATATTGCCCCTCTATAGGCTTC) with *Nco*I and *Bam*H1 restriction enzyme sites. The full-length KaiB gene was amplified using a 5′ primer (5′ CGCGCCATGGATAAAGC CAGAAAGACATACGTCCTCAAGC) and a 3′ primer (5′ CGCGGGATC CTTAAAGATTACTTTGTGCTTCCCAATCTTC), also with *Nco*I and *Bam*H1 restriction sites. PCR products were gel purified, digested with *Nco*I and *Bam*H1 (New England Biolabs), and ligated into the pET32 vector (Novagen). Both KaiA and KaiB were expressed as N-terminal 6-His-thioredoxin fusion proteins in BL21/codonplus *E. coli* cells (Stratagene). The full-length KaiC gene was amplified using a 5′ primer (5′ CGCGCATATGAGTGAGAAAGAGCAGCAGGAA CAAC) and a 3′ primer (5′ CGCGGGATCCTTAGGAATCGGAACCCT GTTTGTCTTC) with *Nde*I and *Bam*H1 restriction sites. The PCR product was gel purified, digested with *Nhe*I and *Bam*H1 (New England Biolabs), and ligated into the pET28 vector. KaiC was expressed with an N-terminal 6-His-tag.

Two KaiB mutants were constructed by introducing Leu to Met mutations at either residue 28 or 69 (QuickChange™, Stratagene). These two sites were chosen based on secondary structure predictions placing them within putative helical regions in KaiB. The primers used were (L28 M) 5′ GTCAGGGCGTTAAAAACA**AT G**AAAAATATTTTAGAACAG and (L69 M) 5′ CGACATTATCAAAGAT T**ATG**CCCCCACCAGTCCGC (mutated base pairs in bold and underlined). KaiB mutant proteins were expressed in BL21/codonplus *Escherichia coli* cells.

Protein expression and purification

KaiA-expressing cells were grown at 37°C in Luria–Bertani broth supplemented with 50 μM carbenicillin in a shaker to an OD_{600} of 0.8. Cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for 4 h (h) at room temperature (RT), then placed at 4°C overnight. Cells were pelleted at 4000 revolutions per minute (rpm), resuspended in buffer (50 mM Tris (pH 7.5), 500 mM NaCl, 5% glycerol, 1 mM PMSF), and sonicated using five 1 min pulses on a Branson Sonifier. The lysed cell solution was centrifuged for 1 h at 17 000 rpm, then the supernatant was mixed with 1 ml of Ni-NTA resin (Qiagen) per litre of lysed cells and gently rocked for 1 h at 4°C. The Ni-NTA resin was then transferred into a column and washed using 100 ml of 50 mM Tris (pH 7.5), 500 mM NaCl, 30 mM imidazole, 5% glycerol. Protein was eluted using 50 mM Tris (pH 7.5), 500 mM NaCl, 250 mM imidazole, 5% glycerol and collected in 1 ml fractions. Concentrated samples were pooled and diluted with 50 mM Tris (pH 7.5), 150 mM NaCl to a final volume of 10 ml. $CaCl_2$ was added to a final concentration of 2.5 mM, followed by 0.5 units of thrombin (Calbiochem). The enzymatic digest proceeded for 2 h at RT, then overnight at 4°C. The sample was subjected to three concentration/dilution cycles using 50 mM Tris (pH 7.5), 150 mM NaCl to lower the imidazole concentration. The sample was then batch-bound onto pre-equilibrated Ni-NTA resin for 1 h at 4°C with gentle shaking and transferred into a column. Flow-through fractions were subjected to three concentration/dilution cycles using 10 mM Tris (pH 7.5), 10 mM Bis–Tris (pH 6.5), 30 mM NaCl, and 0.5 mM TCEP. Afterwards, the sample was loaded onto a Sephadex Q-column (Amersham-Pharmacia). Ion-exchange chromatography was performed using a linear gradient of 0–30% buffer B (Buffer A: 20 mM Bis–Tris (pH 6.5), 30 mM NaCl, 0.5 mM TCEP; Buffer B: 20 mM Bis–Tris (pH 6.5), 1 M NaCl, 0.5 mM TCEP). Fractions (1 ml) were collected and peak fractions identified on a Coomassie-Blue-stained 15% SDS–PAGE gel, pooled, and concentrated. This was followed by buffer exchange on a Superdex 200 column (Amersham-Pharmacia) pre-equilibrated with 20 mM Tris (pH 7.5) and 50 mM NaCl. Fractions (1 ml) were collected and their KaiA content and purity were verified on a gel, then fractions were pooled and concentrated.

For KaiB, the growth, induction, cell lysis, and first nickel column purification steps were performed as described for KaiA. In addition, fractions containing concentrated KaiB protein were pooled and diluted to 10 ml total volume using 50 mM Tris (pH 7.5), 150 mM NaCl, 2.5 mM $CaCl_2$, and 5% glycerol, then 0.5 units of thrombin was added and the protein solution was injected into a Slide-A-Lyser (Pierce). Protein was dialysed against 4 l of buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2.5 mM $CaCl_2$) overnight at 4°C with gentle stirring to remove the 6xHis-Trx fusion tag. The digest was stopped by adding PMSF to a final concentration of 1 mM. The protein solution was mixed with 1 ml of Ni-NTA resin for 1 h at 4°C, packed into a column, and flow-through was collected and concentrated. The protein underwent buffer exchange on a Superdex 200 column (Amersham-Pharmacia) pre-equilibrated with 20 mM Tris (pH 7.5), 50 mM NaCl. Fractions (1 ml) were collected and their KaiB content and purity were checked on a gel, then fractions were pooled and concentrated.

For the expression of the seleno-methionine derivatives (Se-Met), vectors containing the genes for KaiB (L28 M) or KaiB (L69 M) were transformed into B834 (DE3) cells (Novagen). Protein was expressed in M9 media enriched with seleno-methionine and the other 19 amino acids (Ramakrishnan *et al*, 1993). Purification was the same as for the native protein, except that β-mercaptoethanol was added to bring its concentration to 5 mM in the dialysis buffer and subsequent buffer-exchange steps were performed with 0.5 mM TCEP (BioShop) present.

KaiC-expressing cells were grown at 37°C in Luria–Bertani broth supplemented with kanamycin in a shaker until the OD_{600} was 0.8. Induction, cell lysis, and nickel column purification steps were performed as described for KaiB protein, except that all buffers were supplemented with 0.5% SDS. Following elution, samples were pooled and buffer was exchanged three times with 50 mM Tris (pH 7.5), 150 mM NaCl using a 10 kDa cutoff protein concentrator (Millipore). Afterwards, the protein sample was placed on ice for 30 min to precipitate the majority of the SDS, then pelleted via centrifugation at 14 000 rpm for 5 min. The supernatant was collected and again placed on ice. Samples were taken to verify the presence and to determine the amount of KaiC remaining in the soluble fraction.

Size exclusion column chromatography

All protein chromatographic steps were performed using an Äkta FPLC system (Amersham-Pharmacia). Protein samples (500 μl) were prepared as above and size-fractionated by gel filtration on a Superdex 200 (Amersham-Pharmacia) column at 4°C. Protein standards used for calibrating the column were ribonuclease A, chymotrypsin, ovalbumin, albumin, and aldolase (Amersham). Peak samples were loaded onto SDS–PAGE gels and visualized using Coomassie Blue staining. For each protein preparation, two or three independent experiments yielded the same results.

Crystallization, data collection, and processing

KaiA was crystallized by vapour diffusion against 100 mM MES (pH 6.0), 1.1 M $MgSO_4$. The crystals were cryoprotected by adding 20%

glycerol (Sigma) to their mother liquor. To generate a bromine derivative, a native KaiA crystal was soaked for 15 h in a solution containing 100 mM MES (pH 6.0), 1.1 M $MgSO_4$, 160 mM NaBr, and 8% glycerol. No backsoak was performed on the bromine-soaked crystals. Optimal crystallization conditions for KaiB were 100 mM Tris (pH 7.5), 18% (v/v) PEG 400 (Fluka), and for KaiB (L28 M) 100 mM Tris (pH 7.0), 23.5% PEG400, plus 5% 1,6-hexanediol (Fluka).

For data collection, crystals were flash-frozen in a stream of nitrogen at 100 K. All data sets were collected at the Advanced Photon Source beamline 14D (BioCARS) with a MAR345 area detector. MAD data sets ($\lambda_{remote} = 0.9407$ Å, $\lambda_{peak} = 0.9188$ Å, $\lambda_{edge} = 0.9192$ Å) were collected on a bromine-soaked KaiA crystal, whereas the native 2.0 Å resolution data set was obtained at $\lambda = 0.9407$ Å.

A native 2.2 Å resolution data set was collected from a KaiB crystal at $\lambda = 0.9407$ Å. MAD data sets ($\lambda_{remote} = 0.9571$ Å, $\lambda_{peak} = 0.9794$ Å, $\lambda_{edge} = 0.9797$ Å) of 2.5 Å resolution were obtained from a KaiB (L28 M) Se-methionine crystal. Data were reduced using DENZO and scaled using SCALEPACK (Otwinowski and Minor, 1997).

Phase determination

The structure for KaiA was determined applying the MAD method of phasing based on the anomalous signals of bromine atoms diffused into the crystal matrix. Using diffraction data of 2.4 Å resolution, the program package SOLVE (Terwilliger and Berendzen, 1999) found a solution to the heavy atom substructure. Three sites with high occupancies (0.445, 0.667, and 0.477) were found and provided phase information (FOM = 0.51). The corresponding electron density map was then interactively traced using the program O (Jones *et al*, 1991), resulting in a preliminary model of KaiA, which was subsequently refined against the native data using the program CNS, version 1.1 (Brünger *et al*, 1998). Refinement statistics are given in Table I.

The molecular structure of KaiB was determined by the MAD method using KaiB (L28 M) Se-Met protein crystals. Initial positions of the Se atoms were determined with the help of the program SOLVE. Two high-occupancy (0.779 and 0.820) sites were found, one for each subunit of the dimer, and the overall figure of merit was 0.67 for data to 2.5 Å resolution. The density-modified map was then traced manually using the program O. Subsequent refinements were carvried out against the wild-type data set using the program CNS, version 1.1. Refinement statistics are given in Table II. Figures were prepared using the programs SPOCK (Christopher, 1998), MOLSCRIPT (Kraulis, 1991), and RASTER3D (Merritt and Murphy, 1994). Atomic coordinates and structure factors of KaiA and KaiB have been deposited in the RCSB Protein Databank under the accession codes 1R5Q and 1R5P, respectively.

Generation of site-specific mutants to probe protein–protein interactions

The R69A KaiA mutant was chosen due to its conservation and location at the proposed site of interaction between KaiC and KaiA. It was generated by using site-specific mutagenesis. The coding strand sequence of the primer used was (R69A) 5′ GAATTTTC-TAAGCAGCTA**GCA**CTAGAGGGCAGAGGTGAT G (mutation site is noted in bold and underlined). Similarly for KaiB, Arg 23 was mutated to Ala and the coding strand sequence of the primer used was (R23A) 5′ AACACACCAAATTCAGTC**GCG**GCGTTAAAAACACT CAAAAAT (mutation site is noted in bold and underlined). Both KaiA and KaiB mutant proteins were expressed and purified from BL21/codonplus *E. coli* cells as described for wild-type proteins.

6xHis–KaiC interaction assay

The KaiA, KaiB, KaiA (R69A), KaiB (R23A), and 6xHis–KaiC proteins were purified as described above. For each binding assay, varying molar ratios of purified proteins were mixed together in binding solution (50 mM Tris (pH 8.0), 30 mM NaCl, 2.5 mM $MgCl_2$, 5 mM ATP) to a final volume of 400 µl in a 1.5 ml tube. Mixtures were gently rocked on a Nutator (Clay Adams) for 2 h at RT. Ni-NTA agarose resin (Qiagen) was pre-equilibrated using binding solution. In all, 200 µl of a 1:1 slurry of resin and buffer was added to each tube, then the mixture was rocked again for 1 h at RT. Following binding, tubes were centrifuged at 1000 rpm in a microfuge to pellet the resin. The supernatant was carefully removed, resuspended in wash buffer (50 mM Tris (pH 8.0), 30 mM NaCl, 2.5 mM $MgCl_2$, 5 mM ATP, 15 mM imidazole), and transferred into a 0.22 µm spin filtration unit (Millipore). Resin was washed three times using 500 µl of wash buffer and centrifuging the wash through the filter at 1000 rpm for a total wash volume of 1.5 ml. The collection tube was then dried and 500 µl of elution buffer (50 mM Tris (pH 8.0), 30 mM NaCl, 2.5 mM $MgCl_2$, 5 mM ATP, 1 M imidazole) was used to resuspend the resin in the filter unit. The filter unit was centrifuged once more to elute the resin-bound proteins into the collection tubes. Equal volumes of samples from elution and unbound supernatant were loaded onto 10–20% SDS–PAGE gradient gels (NOVEX) and visualized using Coomassie Blue staining.

Table I Data collection and refinement statistics for KaiA

	Native	MAD (Na Br)		
	KaiA	Remote	Peak	Edge
Diffraction data				
Wavelength (Å)	1.0	0.940707	0.9188	0.919226
Resolution (Å)	2.0	2.4	2.4	2.4
Measured reflections (*n*)	101268	111055	115439	119245
Unique reflections (*n*)	9566	5606	5612	5637
Completeness (%)	98.5 (100.0)	98.3 (93.1)	98.6 (98.5)	98.7 (98.7)
*R_{sym} (%)	0.042	0.042	0.062	0.047
Space group	$P6_522$	$P6_522$	$P6_522$	$P6_522$
Molecules in a.u. (*n*)	1	1	1	1
Refinement statistics				
Resolution (Å)	2.0			
Proteins atoms (*n*)	780			
Water molecules (*n*)	38			
Reflections used for R_{free} (*n*)	978			
R_{cryst} (%)	0.242			
R_{free} (%)	0.268			
rmsd bond length	0.01			
rmsd bond angle	1.172			
Average *B*-factor (Å^2)	41.09			

rmsd, root mean square deviation.
*$R_{sym} = \sum|I - \langle I\rangle| / \sum I$, where I is the observed intensity and $\langle I\rangle$ is the average intensity from multiple observations of symmetry-related reflections.

Table II Data collection and refinement statistics for KaiB

	Native	MAD (Se-Met)		
	KaiB	Remote	Peak	Edge
Diffraction data				
Wavelength (Å)	1.0	0.95705	0.97943	0.97971
Resolution (Å)	2.2	2.5	2.5	2.5
Measured reflections (*n*)	67729	70752	74552	73570
Unique reflections (*n*)	10484	10125	10489	10442
Completeness (%)	93.4 (70.1)	96.4 (79.9)	98.4 (91.0)	97.8 (86.5)
$^{*}R_{sym}$ (%)	0.042	0.041	0.048	0.048
Space group	C2	C2	C2	C2
Molecules in a.u. (*n*)	2	2	2	2
Refinement statistics				
Resolution (Å)	2.2			
Proteins atoms (*n*)	1454			
Water molecules (*n*)	67			
Reflections used for R_{free} (*n*)	602			
R_{cryst} (%)	0.209			
R_{free} (%)	0.246			
rmsd bond length	0.012			
rmsd bond angle	1.723			
Average *B*-factor (Å^2)	35.23			

rmsd, root mean square deviation.
$^{*}R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where I is the observed intensity and $\langle I\rangle$ is the average intensity from multiple observations of symmetry-related reflections.

Sequential 6xHis–KaiC interaction assay

For sequential binding assays, 200 µg of 6xHis–KaiC was mixed with 1000 µl 1:1 slurry of Ni-NTA resin and binding solution in a 2.0 ml tube. Solutions used were identical to those in the previous assay. Mixtures were rocked on a Nutator for 1 h at RT. Following incubation, resin was pelleted at 1000 rpm in a microfuge and supernatants were kept. The resin was washed three times with 1 ml binding solution, then resuspended with 500 µl of the same solution. In all, 200 µl of the resuspended 1:1 slurry was taken as a sample and placed into a 0.22 µm spin filtration unit (Millipore). To the remaining 800 µl of 1:1 slurry, 320 µg of KaiA or KaiB was added and mixtures were gently rocked as before. Again, the resin was pelleted and the supernatant was kept. After the resin was washed as before, it was resuspended with 400 µl of binding solution. Again, a 200 µl sample of the 1:1 slurry was taken. To the remaining 600 µl of slurry, 320 µg of the competitor protein (KaiB if KaiA was already added and *vice versa*) was added and the mixtures were gently rocked. The resin was then pelleted and the supernatant was kept. After washing, the resin was resuspended with 300 µl of binding solution. A volume of 200 µl of this slurry was taken as the last sample. All resin samples placed in spin filtration units were washed using 500 µl wash buffer and centrifuged to remove excess solution. After that, resin samples were resuspended in 100 µl elution buffer and filter units were centrifuged to elute the resin-bound proteins into collection tubes. Equal volumes of samples from the elution procedure and from unbound supernatants were loaded onto 10–20% SDS–PAGE gradient gels and visualized using Coomassie Blue staining.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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